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Identification of the key genes involved in the degradation of homocholine by *Pseudomonas* sp. strain A9 by using suppression subtractive hybridization

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ABSTRACT

Microbial transformation of homocholine plays a central role in many biological systems and influence on all kingdoms of life. Here, we used suppression subtractive hybridization (SSH) approach to screen for genes that differentially expressed in response to homocholine by *Pseudomonas* sp. strain A9 and to gain deep acknowledge about the gene expression and sequences of homocholine degrading enzymes. Twenty-seven differentially expressed genes were identified and were found to involve in the uptake and metabolism of homocholine as well as physiological responses of strain A9 to this compound. Of them, fragments of homocholine dehydrogenase (hcdH), β-alanine betaine aldehyde dehydrogenase (bABALDH), β-alaninebetaine CoA transferase (hcdD), 3-hydroxypropionate dehydrogenase (hcdB), and malonate semialdehyde dehydrogenase (hcdC) genes were detected. After excessive experiments of PCR and sequencing, the full-length sequences of these key genes were identified. Interestingly, a complete sequence of a unique gene cluster (6.2 kbp) of hcd (homocholine degrading) genes that contain the genes hcdD, hcdB, hcdC, and hcdR was obtained. The sequence information of these essential genes will enhance our understanding of homocholine catabolic pathway in microorganisms and will help in identifying better inhibitors or activators of these enzymes to either improve or suppress their activity depending on the importance of the formed metabolite.

1. Introduction

Homocholine (3-*N*-trimethylamino-1-propanol) is a quaternary ammonium compound that shares high structural similarity with choline, in which the carbon chain is lengthened by one CH_2-group [1]. It also has a great resemblance to choline in many aspects of cholinergic metabolisms [2]. In the mammalian brain, both homocholine and choline are transported into nerve terminals by the same high-affinity uptake mechanisms [3], where they are acetylated by a membrane-associated form of choline acetyltransferase and released respectively as acetylhomocholine and acetylcholine by a calcium-dependent process during depolarization [4,5]. Consequently, homocholine acts as a competitive inhibitor of choline acetylation by the soluble form of choline acetyltransferase, while both compounds serve as competitive alternative substrates for the membrane-associated form of choline acetyltransferase [6]. Due to its high similarity to choline in uptake and acetylation, homocholine motif or head group was incorporated into various types of synthetic and natural compounds used as pharmaceutical drugs and insecticides. Of them, methyllycaconitine 1, is a natural compound contains an acetylated homocholine motif that competitively blocks α7 nicotinic acetylcholine receptor subtype [7,8]. This compound is considered as a powerful drug to cure various types of brain-related diseases such as pain, Alzheimer's disease, Parkinson's disease, schizophrenia and depression [9] besides its insecticidal activity. In addition, homocholine motif or head group are integrated into a synthetic drug known as erufosine (erucylphospho-N,N,N-trimethyl propylammonium, erucylphosphohomocholine, ErPC3) that targeting

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various types of cancer and bone marrow cells [10,11]. Although the metabolism of choline in mammalian and microbial cells intensively investigated, studies on homocholine metabolisms are scarce [1,12–14] and further investigations on the microbial transformation of this important compound are still required. Although homocholine is not widely distributed in nature, the recently increased trials of homocholine containing compounds in pharmaceutical drugs and insecticides necessitate the in-depth studies of its microbial transformation [1]. The aerobic and anaerobic microbial transformations of choline, homocholine, and L-carnitine had led to the formation of essential intermediate metabolites such as trimethylamine (TMA), glycine betaine, β-alanine betaine, and γ-butyrobetaine [1,13,15–19]. Glycine betaine, β-alanine betaine, and γ-butyrobetaine are known as osmoregulators that allow the organism to survive under harsh environmental conditions [19–25]. Whereas, TMA participates in the development of cardiovascular diseases [26,27], nonalcoholic fatty liver disease [28], and metabolic disorder trimethylaminuria [29] as well as it could contribute to the global carbon and nitrogen cycle [1,30]. Consequently, the microbial transformations of choline, homocholine, and L-carnitine play a significant role in many biological systems and impact on all kingdoms of life [1]. Our recent proteomic study discovered most of the proteins involved in the degradation of homocholine by *Pseudomonas* sp. strain A9 [1]. However, the complete understanding of the essential genes involved in the transformation pathway of homocholine in microorganisms remains unclear. Therefore, in the current study, we used suppression subtractive hybridization (SSH) techniques to screen and identified the genes contribute to the degradation of homocholine in *Pseudomonas* sp. strain A9. In this study, we identified most of the differentially expressed genes in cells lysate of *Pseudomonas* sp. strain A9 grown on homocholine, and we found that the identified genes cover a diverse range of metabolic processes. In addition, we sequenced and analyzed the genes of the key enzymes contribute to the degradation of homocholine by strain A9.

2. Materials and methods

2.1. Bacterial strain and culturing conditions

The bacterial isolate *Pseudomonas* sp. A9 that had isolated previously from soil [13] were stored either in basal-homocholine slant media at 4 °C or in 50% glycerol and stored in a freezer at −80 °C. Inoculum of bacterial samples of *Pseudomonas* sp. strain A9 stored at −80 °C and 4 °C were inoculated into 5 mL basal medium containing 2 g/L KH_2PO_4; 2 g/L K_2HPO_4; 0.5 g/L $MgSO_4{\cdot}7H_2O$; 0.5 g/L yeast extract; and 1 g/L polypeptone (pH 7.0) containing either 10 g/L homocholine or 10 g/L citrate as carbon and energy sources. The bacterial culture was incubated at 26 °C for 24 h on a reciprocal shaker at 140 rpm. When the growth reach the turbidity of 2.0 (T660 nm = 2.0), about 100 μL inoculums of this culture was transferred to 100 mL/500 mL flask of fresh basal media containing either 10 g/L homocholine or 10 g/L citrate and incubated at 26 °C. The bacterial cells were collected at the mid-exponential phase (15 h), and the cultures were then harvested by centrifugation at 10,000 × *g* for 20 min at 4 °C and wash twice with 0.85% KCl solution. The collected cells were used immediately for RNA extraction using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA).

2.2. Total RNA extraction and estimation

The bacterial cells were suspended in 2 mL of protoplast buffer (15 mM Tris-HCl pH 8.0, 8 mM EDTA, and 0.45 M sucrose) and kept on ice. Immediately 20 μL of freshly prepared lysozyme (50 mg/ mL) was added, mixed well, and kept on ice for 15 min. The protoplast was then collected by centrifugation at 5000 × *g* for 15 min at 4 °C. Then, the protoplast was suspended in 0.5 mL of gram-negative lysing solution (10 mM tris-HCl pH 8.0, 10 mM NaCl, 1 mM sodium citrate, and 1.5% SDS) and incubated at 37 °C for 5 min. After that, 2 mL of TRIzol reagent was added, and total RNA was extracted according to the manufacturer's instructions. The quantity of the extracted RNA was measured by using SmartSpecTM plus spectrophotometer (Bio-Rad Lab, Hercules, California, USA). Before measuring, the total RNA was dissolved in RNase-free water and diluted 100 times with 10 mM tris-HCl buffer, pH 7.5. Then, the spectrophotometer was calibrated to zero with the blank solution of 10 mM tris-HCl, pH 7.5. After that, triplicate samples of each RNA sample were measured at 260 and 280 nm. In one hand, the quality of RNA was estimated with denaturing agarose gel electrophoresis. Briefly, 1% gel was casted using the following; 18%formaldehyde, 20 x MOPS buffer (pH 7.3), and agarose in RNase-free water. The RNA samples (5 μg) were added to 20 μL of sample loading buffer of 5x MOPS buffer (pH 7.3), 17.5% formaldehyde, 50% formamide, and 2x BPB. The mixture was heated at 65 °C for 15 min and immediately put on the ice. The samples were then loaded into the gel and separation was carried out using 1x MOPS buffer (pH 7.3) at 50 V for 1 h. The gel was then stained with ethidium bromide (20 μg) in 1x MOPS buffer pH 7.3 for 20 min, and then destaining was performed in Mili-Q water (20 min × times). The intact RNA was treated with DNase to remove contaminants.

2.3. DNase treatment and RNA purification

Contaminating DNA was removed by using DNA-free DNase treatment and removal reagents (Ambion, Austin, TX, USA). Briefly, to 20 μL sample about 2 μL of DNase buffer and 1 μL of DNase were added and mixed gently. The mixture was incubated at 37 °C for 30 min, and then 2 μL of DNase inactivation solution was added and mixed well. After 2 min incubation at room temperature, the mixture was centrifuged at 10,000 × *g* for 1.5 min and the supernatant was transferred to new tube. The absence of contaminating DNA was observed by both TAE agarose gel and MOPS/formaldehyde agarose gel. Total RNA was purified by using RNase mini kit as described in the instruction manual (Qiagen, Tokyo, Japan). The purified RNA was further precipitated with ethanol as mentioned in the kit. The purity of RNA was checked by both TAE and MOPS/formaldehyde agarose gels, whereas, the quantity of RNA was estimated by spectrophotometric analysis at 260/280 nm. Throughout the study, to avoid or minimize the contamination the proper microbiological aseptic techniques were applied.

2.4. Microbe express mRNA enrichment

About 40 μg of the total RNA extracted from homocholine (tester) growing cells and 40 μg RNA extracted from citrate (driver) growing cells of strain A9 were used for mRNA purification using MICROB Express ™ bacterial mRNA purification kit (Ambion, Austin, TX, USA) following the instruction manual. To ensure the purity and enrichment of mRNA, at least three rounds of purification steps were carried out and after each round, both the quantity and quality of mRNA were analyzed. The disappearance of the 16SrRNA and 23SrRNA bands combined with the increase of the intensity of small size bands and smearing are indicators for the purity of the mRNA. The high ratio of A260/280 nm is another indicator for the purity of mRNA. Multiple preparations of each substrate were pooled before ethanol precipitation. The purified mRNA was concentrated by precipitation with ethanol and resolved in a small volume of RNase-free water and used for the synthesis of first strand cDNA.

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